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Effects on Fitness and Behavior of Monarch Butterfly Larvae Exposed to a Combination of *Cry1Ab*-Expressing Corn Anthers and Pollen

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ABSTRACT Anthers and pollen from corn, *Zea mays* L., expressing *Bacillus thuringiensis* (Bt)-derived protein frequently fall onto common milkweed, *Asclepias syriaca* L., growing in and near corn fields. Previous studies have shown that, alone, Bt anthers do not pose a significant risk to the monarch butterfly, *Danaus plexippus* L. To examine how exposure to a combination of Bt anthers and pollen affects larval fitness and behavior, three studies were conducted. A laboratory study using severed leaves in petri dishes and two studies with caged milkweed plants (tropical milkweed, *Asclepias curassavica* L., and common milkweed) in an environmentally controlled rearing room. In the petri dish bioassay, additive effects of Bt anthers and pollen were detected. The data suggest that the additive effects may be attributed to behavioral changes in larval feeding such as detecting and avoiding Bt anthers. An additive effect also was seen in both cage studies. In the common milkweed cage study, larvae exposed to Bt anthers and pollen took 1.8 d longer to develop and pupae weighed 6.4% less than those exposed to non-Bt anthers and pollen. These effects are similar to those found in a previous study with naturally deposited levels of Bt anthers and pollen, even though the anther levels we tested were two to three times greater. Despite these effects, when put into the context of risk, Bt corn is not likely to pose a significant risk to the monarch butterfly population in North America.

KEY WORDS transgenic corn, nontarget, risk assessment, *Danaus plexippus*

CORN, *Zea mays* L., pollen expressing *Bacillus thuringiensis* (Bt)-derived protein is deposited onto leaves of common milkweed, *Asclepias syriaca* L., in Bt corn fields during anthesis (Pleasants et al. 2001). A laboratory study by Losey et al. (1999) suggested that larvae of the monarch butterfly, *Danaus plexippus* L., may be adversely affected by consuming milkweed leaves dusted with Bt corn pollen. Extensive laboratory and field studies were conducted, and a risk assessment concluded that the impact of Bt corn pollen from current commercial corn hybrids on monarch butterfly populations is negligible (Hellmich et al. 2001, Oberhauser et al. 2001, Pleasants et al. 2001, Sears et al. 2001, Stanley-Horn et al. 2001, Zangerl et al. 2001). The risk assessment did not address the potential risk from anthers, which also fall onto milkweed leaves and contain Bt toxin. Adverse effects of anther ingestion have been documented in the laboratory (Hellmich et al. 2001, Anderson et al. 2004). However, field studies show that toxic anther densities are uncommon on milkweed leaves during anthesis and field exposure to a common anther density (five anthers per leaf) showed no adverse effects on monarch butterflies (Anderson et al. 2004). Based on low probability of exposure to toxic densities, Bt anthers alone are not likely to pose a significant risk to monarch butterflies. However, anthers do not occur alone in the field but rather in combination with pollen. Studies by Dively et al. (2004) detected adverse effects on monarch butterflies exposed to naturally deposited levels of Bt anthers and pollen, but they did not separate the effects caused by pollen versus anthers. Also, the mean anther densities reported in these studies (1.1–1.8 anthers/leaf) were lower than those reported on milkweed leaves in corn fields by Anderson et al. (2004) (3–5 anthers/leaf). If the anther densities had been at levels found by Anderson et al. (2004), would the effects have been more severe or would similar adverse effects have been seen? To address this question and to examine how exposure to Bt anthers and pollen separately and in combination affects larval development, survival and behavior, three studies were conducted: a laboratory study using milkweed leaf disks in petri dishes and two studies with caged milkweed plants (tropical milkweed, *Asclepias curassavica* L., and common milkweed) in an environmentally controlled rearing room.

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Materials and Methods

General Protocol

Monarch butterfly larvae were from a colony established with ≈1,200 eggs collected from 25 locations in and near Ames, IA, from 21 May to 19 June 2003. Larvae were maintained on fresh surface-sterilized common milkweed leaves. Leaves were sterilized in a 0.6% solution of sodium hypochlorite for 10 min followed by three 1-min rinses in a salad spinner with tap water. All adults tested negative for the presence of the protozoan parasite *Ophryocystis elektroscirrha* using the Scotch tape technique used by Altizer et al. (2000).

Petri Dish Bioassay

Monarch butterfly neonates were exposed to common milkweed leaf disks (21 mm diameter) to which the following treatments were applied: (1) Bt anthers, (2) non-Bt anthers, (3) Bt pollen, (4) non-Bt pollen, (5) Bt anthers and pollen, (6) non-Bt anthers and pollen, and (7) no anthers or pollen. The petri dish arenas were prepared as follows: (1) two layers of solidified agar (2.5% wt:vol, 1.5 and 2.5 mm thickness) were prepared in separate culture plates, (2) the 1.5-mm layer was removed for its plate and put over the 2.5-mm layer, (3) a no. 13 cork borer was used to produce one 18-mm hole through both layers of agar in the center of the dish, and (4) the top agar layer was pulled back and a 21-mm leaf disk (no. 15 cork borer), with a portion of the natural leaf edge intact, was put over the hole in the bottom layer, after which the top layer was repositioned to seal the disk between the agar layers. This setup delayed leaf disk dehydration. The natural leaf edge was positioned in a manner that allowed the larva to move freely between the top and bottom surfaces of the leaf.

Anthers and pollen were collected and processed using the same methods as the Iowa studies in Hellmich et al. (2001). Anthers and pollen were from Bt hybrid 38G17Bt (MON810 event; Pioneer Hi-Bred International, Johnston, IA) or its near isoline 3893 (Pioneer Hi-Bred International). For treatments with anthers, whole anthers (examined under a dissecting microscope to ensure they were undamaged) were placed on the milkweed leaf disks at a density of 0.6 anther/cm^2 (≈30 anthers per whole common milkweed leaf). Anthers had dehisced; however, small amounts of pollen remained in some anthers. Pollen was applied, and the mean density was estimated using the same methods as the Iowa studies in Hellmich et al. (2001). The target density was 170 pollen grains/cm^2, the mean pollen density found on milkweed leaves in corn fields by Pleasants et al. (2001). The mean density achieved was 171 ± 49 pollen grains/cm^2. Using a camel-hair brush, one monarch butterfly neonate was placed in each dish.

After 4 d, larvae were weighed, and using a dissecting microscope with an eyepiece reticle grid, the area of leaf and anther material consumed (square millimeters) during the first 4 d of development was counted. Larvae were transferred to larger petri dishes (100 by 15-mm Fisherbrand; Fisher, Pittsburgh, PA) coated with a thin layer of agar (≈1 mm) on the inner surfaces to reduce static electricity and keep anthers on the leaf surface. A milkweed leaf disk (7.8 cm diameter) was placed in each petri dish. For treatments with anthers, sufficient anthers were added to maintain the same density as the smaller dishes (0.6 anther/cm^2). Leaf material with the appropriate treatment applied was replaced every other day, and the area of anther material consumed (square millimeters) was estimated until day 10. After 10 d of exposure to the treatments, larvae were weighed and transferred to inverted 236 ml (8 oz) clear plastic cups (Waddington North America, Chlemsford, MA) placed on large petri dish lids and fed milkweed leaves with no anthers or pollen until pupation. All stages of the experiment were incubated at 25°C, 8-h scotophase, and 60% RH.

The experiment was conducted in two temporal blocks. Each treatment was replicated 20 times in block 1 and 25 times in block 2. Data recorded included 4-d leaf feeding, 4- and 10-d larval weight, number of days to pupation and eclosion, pupal weight, and anther feeding. Anther feeding data were grouped into two time periods based on larval susceptibility to Bt toxin: the first 4 d of exposure (when larvae are most susceptible to Bt) and the last 6 d of exposure (when larvae are less susceptible to Bt toxin; Hellmich et al. 2001, Anderson et al. 2004). Separate analyses of variance (ANOVAs) were used to test for treatment effects on measurements of feeding and development with block treated as a random effect (PROC MIXED; SAS Institute 1999). If the *F*-tests indicated treatment differences, a priori linear contrasts were conducted to test for differences between Bt and non-Bt treatments with the same tissue types (e.g., contrast Bt anthers and non-Bt anthers). The number of larvae surviving to pupation and eclosion in each treatment were treated as a binomial experiment with each larva surviving representing a single successful trial. The probability of success for a treatment, π_i, was equal to the number of larvae surviving in a treatment divided by the number of replicates per trial ($n = 45$). To test whether Bt tissues reduced survival, the two binomial proportions (Bt and non-Bt of the same tissue type) were compared using the normal approximation with the alternative hypothesis $\pi_{\text{non-Bt}} - \pi_{\text{Bt}} > 0$ (Ott 1993). This analysis also was conducted on anther feeding data for the first 4 d of exposure and the last 6 d of exposure for treatments with the same tissue types to test whether the presence of Bt affected the proportion of larvae feeding on anthers.

Cage Studies

Two cage experiments were conducted in an environmentally controlled room (25°C, 8-h scotophase, and 60% RH): one with potted, greenhouse-grown tropical milkweed and one with fresh cut, field-collected common milkweed. Both studies had the same

seven treatments as the petri dish bioassay replicated 10 times each. Anthers and pollen were from Bt hybrid N58-D1 (Bt11 event; Syngenta Seeds, Golden Valley, MN) or its near isoline N58-F4 (Syngenta Seeds).

For the tropical milkweed experiment, a 10 by 10-cm pot (Nursery Supplies, Fairless Hills, PA) containing a tropical milkweed plant (≈1 m in height) was placed in each 19-liter (5 gal) bucket. A tomato cage was placed into each bucket and a no-see-um mesh bag (Arrowhead Fabric Outlet, Duluth, MN) was used to enclose the cage, exclude predators, and keep the larvae from moving off the plant. For treatments with anthers, five anthers were placed on each leaf. This density represented the peak mean anther density found on milkweed leaves in and near cornfields during anthesis by Anderson et al. (2004). For treatments with pollen, two freshly collected corn tassels were shaken over the plant. A 1.5-m tall, 0.9-m wide plastic cylinder was used to surround the plant and keep neighboring plants from receiving pollen, while a funnel (60/30-cm top/bottom diameter) and 60 USA Standard Test Sieve (Newark Wire Cloth Co., Newark, NJ) were held over the plant to keep additional anthers from falling onto the plant. One leaf was removed from the middle of each plant to estimate pollen density using the same methods as the Iowa studies in Hellmich et al. (2001). The mean pollen density was 228 ± 130 pollen grains/cm^2. Three monarch butterfly neonates were placed on each plant.

For the common milkweed experiment, milkweed plants from a nonagricultural area were cut at the ground level, placed in water, transported to the rearing room, and placed immediately into cages. Cages consisted of a 19-liter (5 gal) bucket with a 185-ml pill vial (50 dram) glued inside on the bottom. A 40-mm foam test tube plug (Daigger, Vernon Hills, IL) was cut to allow the milkweed stem to slide through and kept the stem upright when placed into the pill vial. Vials were checked daily, and water was replenished as needed using a 10-ml pipette attached to a 160-ml syringe (Becton Dickson & Co., Franklin Lake, NJ) with a 5-mm (3/16 in) diameter plastic tube (Nalge Co., Rochester, NY). Using this apparatus, vials could be filled without disturbing the anthers, pollen, or larvae on the plant. Anthers, pollen, and larvae were applied as described above. The mean pollen density was 202 ± 101 pollen grains/cm^2. Before the lid was secured to the bucket, a hole was cut and covered with no-see-um mesh for ventilation. Milkweed plants were replaced every 3 d, and all surviving larvae were moved to the new plant.

For both studies, larvae remained in the cage for 11 d, at which time they were removed, weighed, and fed untreated leaves until pupation. Data recorded for individual larvae included 11-d larval weight, days to pupation and eclosion, and pupal weight. Potential effects of treatment on each of the four variables were tested with ANOVA (PROC MIXED; SAS Institute 1999). Cages were considered the experimental unit and individual larvae were treated as subsamples using a WEIGHT statement, with the weight equal to the number of individuals remaining from each cage when data were recorded. Because an interaction between treatment and milkweed type was found in a combined analysis, data were analyzed separately for each milkweed type. For one variable (11-d larval weight), log-transformation was used to meet assumptions of normality. If the overall ANOVA indicated treatment differences, a priori linear contrasts were conducted to test for differences between Bt and non-Bt treatments with the same tissue types (e.g., contrast Bt anthers and non-Bt anthers). Data collected on individual larvae also provided information on survival to pupation and eclosion, but because of the small number of larvae per cage (≤3), percentage survival to pupation and eclosion could not be treated as continuous variables. Instead, survival was treated as a binomial experiment with each larva surviving representing a single successful trial. The probability of success for a treatment, π_i, was equal to the number of larvae surviving in a treatment divided by the number of replicates per trial ($n = 30$). To test whether Bt tissues reduced survival, the two binomial proportions (Bt and non-Bt of the same tissue type) were compared using the normal approximation with the alternative hypothesis $\pi_{\text{non-Bt}} - \pi_{\text{Bt}} > 0$ (Ott 1993).

Comparison of Milkweed Species

Leaf area and density were compared between the two milkweed leaf species (tropical and common). Fifty milkweed leaf disks (≈2 cm^2) were cut from each milkweed species using a no. 11 cork borer (1.5 cm diameter). Leaf disks were dried at 45°C for 48 h and weighed using an analytical balance. To estimate the mean area of tropical and common milkweed leaves, 25 leaves were randomly selected from tropical milkweed plants grown in a greenhouse and common milkweed plants growing in and near cornfields. The length and width of each leaf were measured and used to calculate an estimate of the area. Data were subject to ANOVA with Tukey's studentized range test used to separate means ($P \leq 0.05$; SAS Institute 1999).

Results

Petri Dish Bioassays

Short-term Exposure. After 4 d of exposure, there were significant differences in leaf feeding among treatments ($F_{(6,6)} = 4.6$, $P = 0.043$; Fig. 1). Linear contrasts did not detect differences in leaf feeding for larvae exposed to Bt or non-Bt anthers or between larvae exposed to Bt pollen or non-Bt pollen. However, larvae exposed to a combination of Bt anthers and pollen fed significantly less than larvae exposed to non-Bt anthers and pollen. ($F_{(1,6)} = 7.3$, $P = 0.036$). During the first 4 d of exposure, the area of anther material consumed was low across all treatments (Fig. 2). Larvae exposed to Bt anthers and pollen consumed a smaller area of anther tissue than larvae

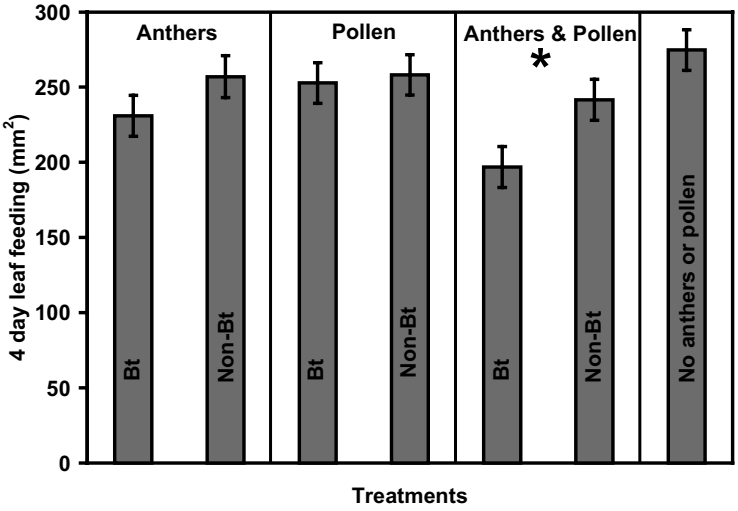

Fig. 1. Mean ± SE 4-d leaf feeding for monarch butterfly larvae exposed to seven combinations of Bt and non-Bt anthers and pollen on milkweed leaf disks in the laboratory (anther density, 0.6 anther/cm^2; pollen density, 171 ± 49 pollen grains/cm^2). *Linear contrast detected differences between Bt and non-Bt treatments with the same tissue types.

exposed to non-Bt anthers and pollen. During the first 4 d of exposure, the proportion of larvae that fed on anthers was low across all treatments (Bt anthers = 0.02, non-Bt anthers = 0.11, Bt anthers and pollen = 0.09, non-Bt anthers and pollen = 0.29). The proportion of larvae that fed on anthers was low in treatments with Bt regardless of whether pollen was present. When only anthers were present, there was no difference between the proportions of larvae feeding on Bt anthers versus non-Bt anthers. When anthers and pollen were present, a greater proportion of larvae fed on non-Bt anthers than Bt anthers ($z = 2.42$; $P = 0.008$).

Long-Term Exposure. After 10 d of exposure, a smaller proportion of larvae survived to pupation and eclosion in the Bt anthers and pollen treatment (0.64 and 0.53, respectively) than the non-Bt anthers and pollen treatment (0.80 and 0.84, respectively; survival to pupation: $z = 2.17$, $P = 0.015$; survival to eclosion: $z = 2.94$, $P = 0.002$). The proportion of larvae surviving to pupation or eclosion was not different for larvae exposed to Bt anther versus non-Bt anthers or Bt pollen versus non-Bt pollen. During the last 6 d of exposure, there were no differences among treatments for the area of anther material consumed (Fig. 2). The proportion of larvae that consumed anther tissue was high for all treatments (Bt anthers = 0.75, non-Bt anthers = 0.86, Bt anthers and pollen = 0.73, non-Bt anthers and pollen = 0.89). When only anthers were present, the presence of Bt toxin did not seem to affect the frequency of anther feeding; however, when both anthers and pollen were present, a smaller proportion of larvae fed on Bt anthers tissue ($z = 1.83$, $P = 0.034$). No significant differences were detected among treatments for 4- or 10-d larval weight, days to pupation, pupal weight, days to eclosion, or survival to eclosion (Table 1).

Cage Studies

In the tropical milkweed experiment, larvae exposed to Bt anthers took 1.4 and 1.7 d longer to pupate and eclose, respectively, than larvae exposed to non-Bt anthers (Fig. 3). Larvae exposed to Bt pollen weighed less and took 1.5 and 2.0 d longer, respectively, to pupate and eclose compared with larvae from the non-Bt pollen treatment (Fig. 3). Larvae exposed to a combination of Bt anthers and pollen weighed less and took 3.6 and 4.2 d longer to pupate and eclose, respectively (Fig. 3). The proportion of larvae surviving to pupation in the Bt anthers and pollen treatment (0.53) also was lower than the non-Bt anthers and pollen treatment (0.80; $z = 2.19$, $P = 0.014$).

In the common milkweed experiment, no differences were detected between larvae exposed to Bt or non-Bt anthers. Larvae exposed to Bt pollen took 1.9 d longer to pupate than larvae exposed to non-Bt pollen (Fig. 4). Larvae exposed to a combination of Bt anthers and pollen took 1.8 d longer to pupate and had reduced pupal weights compared with those exposed to a combination of non-Bt anthers and pollen. There were no differences in the proportion of larvae surviving to pupation or eclosion for any of the Bt/non-Bt tissue comparisons.

Comparison of Milkweed Species

The 2-cm^2 common milkweed leaf disks had greater mass than the tropical milkweed leaf disks: 6.8 ± 0.9 and 3.6 ± 0.5 (SE) mg, respectively ($F_{(1,98)} = 490.4$, $P < 0.001$). The mean leaf area of common milkweed was greater than tropical milkweed: 54.8 ± 0.8 and 35.6 ± 2.9 cm^2, respectively ($F_{(1,48)} = 19.0$, $P < 0.001$).

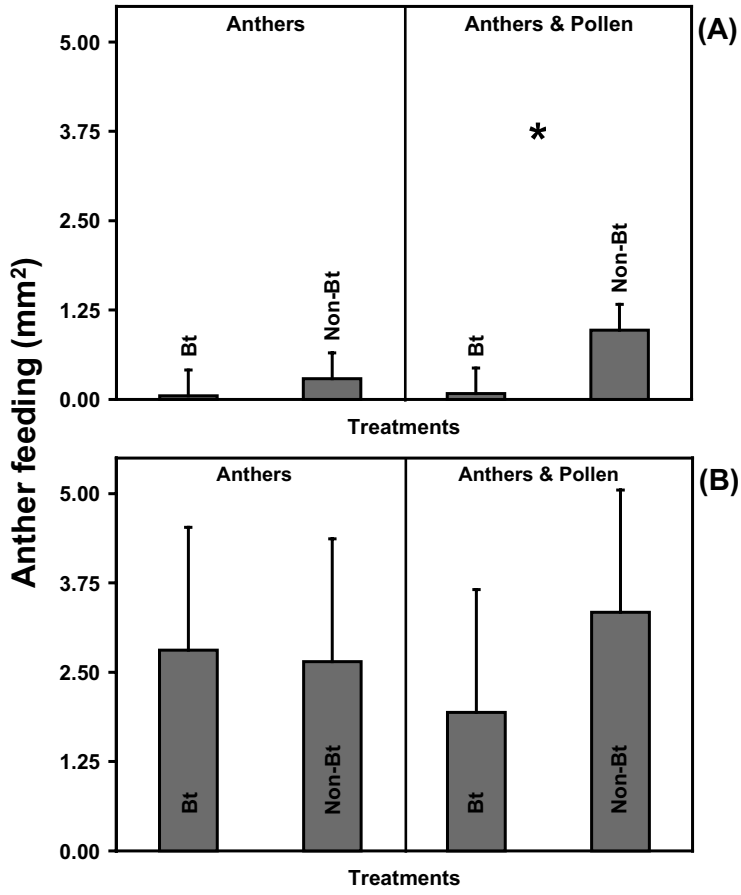

Fig. 2. Mean ± SE anther feeding for monarch butterfly larvae exposed to only anthers (Bt or non-Bt) or a combination of anthers and pollen (Bt or non-Bt) on milkweed leaf disks in the laboratory (anther density, 0.6 anther/cm^2; pollen density, 171 ± 49 pollen grains/cm^2). (A) Mean anther tissue consumed during the first 4 d of a 10-d exposure (starting with neonate larvae). (B) Mean anther tissue consumed during the last 6 d of the 10-d exposure. *Linear contrast detected differences between Bt and non-Bt treatments with the same tissue types.

Discussion

Petri Dish Bioassay

Short-Term Exposure. Results support the hypothesis that exposure to Bt pollen and anthers have additive effects on monarch butterfly larvae. While contrasts between Bt anthers and non-Bt anthers and Bt pollen and non-Bt pollen revealed no detectable effect of the Bt toxin, larvae exposed to a combination of Bt anthers and pollen consumed less leaf area than those exposed to non-Bt anthers and pollen (Fig. 1). The lack of differences detected between pollen-only treatments was expected because pollen levels were below the no-observable-effects level for short-term

Table 1. Petri dish study exposing monarch butterfly larvae to anthers and pollen separately and in combination

	Treatments							$F_{(df)}$	P
	Anthers		Pollen		Anthers and pollen		None		
	Bt	Non-Bt	Bt	Non-Bt	Bt	Non-Bt			
Larval wt 4 d[a]	14.1	18.1	19.0	18.2	13.3	18.7	18.6	$2.40_{(6,6)}$	0.155
Larval wt 10 d[a]	742.6	811.8	788.0	758.1	717.5	794.4	863.7	$2.67_{(6,6)}$	0.128
Days to pupation	13.0	12.8	12.7	13.0	13.3	12.7	12.6	$2.06_{(6,6)}$	0.200
Pupal wt (mg)	1204.9	1179.4	1143.1	1221.6	1175.4	1187.0	1181.9	$1.10_{(6,6)}$	0.457
Days to eclosion	26.1	25.7	25.7	26.1	26.6	25.9	25.8	$2.35_{(6,6)}$	0.161

Anthers and pollen were 38G17Bt (MON810 event) and near isoline 3893 (Pioneer Hi-Bred International).
Anther and pollen densities were 0.6 anther/cm^2 and 171 ± 49 pollen grains/cm^2.
[a] Larval weights (mg) were log-transformed before analysis; back-transformed means shown here.

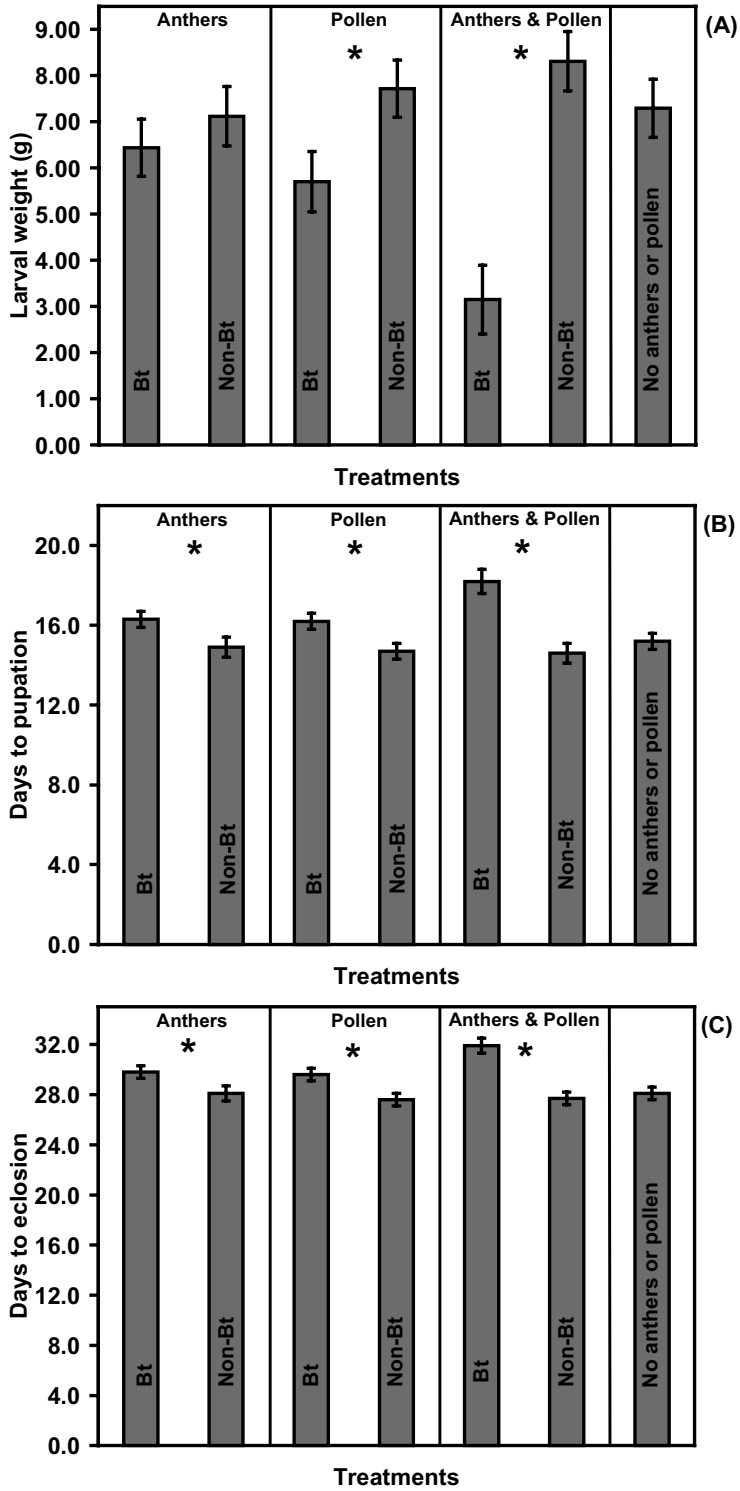

Fig. 3. Effects on (A) larval weight, (B) days to pupation, and (C) days to eclosion in the tropical milkweed cage study exposing monarch butterfly larvae to anthers and pollen separately and in combination. Anther density was five per leaf and pollen density was 228 ± 130 grains/cm^2. Larval weights (A) were log-transformed before analysis; back-transformed means are shown here. *Linear contrast detected differences between Bt and non-Bt treatments with the same tissue types.

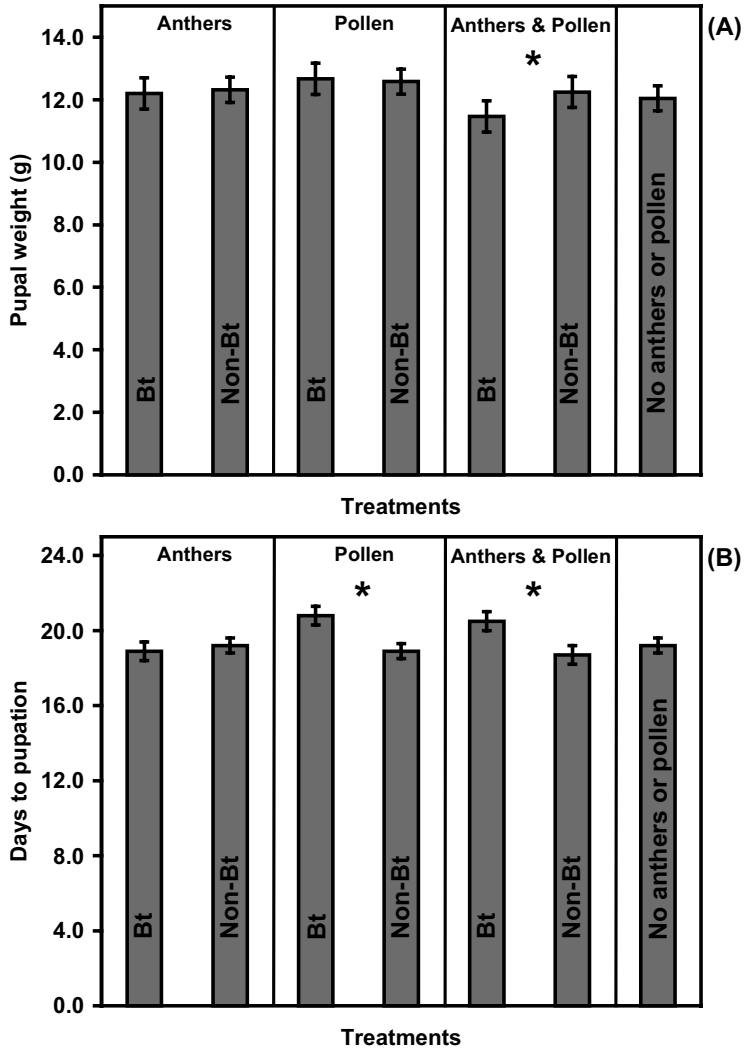

Fig. 4. Effects on (A) pupal weight and (B) days to pupation in the common milkweed cage study exposing monarch butterfly larvae to anthers and pollen separately and in combination. Anther density was five per leaf and pollen density was 202 ± 101 grains/cm^2. *Linear contrast detected differences between Bt and non-Bt treatments with the same tissue types.

exposure to Bt pollen (Hellmich et al. 2001). The mean area of anther tissue consumed in the anther-only treatments was low during the first 4 d and probably explains why differences were not detected between the anther-only treatments (Fig. 2).

Examination of data on the proportion of larvae feeding on anthers revealed a possible behavioral mechanism for the additive effects seen in the laboratory. When both anthers and pollen were present, a smaller proportion of larvae fed on anthers in the Bt treatment than the non-Bt treatment. Larvae could be detecting Bt anthers, and avoiding them or the decreased anther feeding may be a side effect of increased intoxication from consumption of Bt pollen. Consumption of sublethal doses of Bt pollen could lead to decreased leaf feeding, fewer encounters with anthers, and consequently, less anther feeding. Data on 4-d leaf feeding support this hypothesis, showing that larvae exposed to Bt anthers and pollen fed on less milkweed leaf tissue than larvae exposed to non-Bt anthers and pollen. If the effects seen on anther feeding were caused by decreased leaf feeding as a result of Bt pollen consumption, one would expect to see difference in leaf feeding between the Bt pollen and non-Bt pollen treatments as well. However, there was no difference in leaf feeding between the pollen only treatments. Therefore, it is not Bt pollen consumption alone that is resulting in less leaf feeding. Perhaps the presence of anthers adds another negative stimulus to feeding. The concentration of Bt in anther tissue is greater than pollen (U.S. EPA 2001); therefore, consumption of even a small amount of Bt anther tissue could lead to increased Bt intoxication. Because such a small proportion of larvae actually consumed Bt

anther tissues in the first 4 d or development (Bt anthers only = 0.02, Bt anthers and pollen = 0.09), it is likely that there is some other behavioral mechanism affecting leaf feeding. Perhaps the presence of Bt anthers causes behavioral changes such as increased searching for an area with no anthers resulting in less leaf feeding. Studies on larval feeding behavior using a video-tracking system have shown effects on feeding behavior in the presence of Bt anthers, suggesting some mechanism of detection and avoidance of Bt anthers by monarch butterfly larvae (Anderson 2004). A more direct method of testing the ability of monarch larvae to detect Bt toxin would be to monitor neural signals generated from larval receptors in response to the presence of Bt toxin; such studies have been conducted with other lepidopteran species with substances other than Bt (Schoonhoven and Van Loon 2002).

Long-Term Exposure. During the last 6 d of exposure, there also was evidence to support the hypothesis that exposure to Bt pollen and anthers have additive effects on monarch larvae. Even with long-term exposure (10 d) to anthers only or pollen only, differences were not detected between the Bt anther and non-Bt anther treatments or the Bt pollen and non-Bt pollen treatments. However, with a combination of anthers and pollen present, reduced survival to pupation and eclosion was detected between the Bt and non-Bt treatments. The area of anther material consumed was high across all treatments, confirming the conclusion of previous studies that older monarch butterfly larvae are more tolerant of Bt toxin (Hellmich et al. 2001, Anderson et al. 2004).

Cage Studies

The cage studies were conducted to simulate more realistic exposure to anthers and pollen separately and in combination. Additive effects also were seen in both cage studies. With common milkweed, no differences were detected in pupal weight when comparing larvae exposed to Bt anthers and non-Bt anthers and Bt-pollen and non-Bt pollen. There were no differences in days to pupation for larvae exposed to anthers only, but larvae exposed to Bt pollen did take longer to pupate than those exposed to non-Bt pollen, suggesting that exposure to pollen may play a more significant role in toxicity (Fig. 4). When larvae were exposed to a combination of Bt anthers and pollen, the resulting pupae weighed less and took longer to eclose than those exposed to non-Bt anthers and pollen (Fig. 4). With tropical milkweed, delays in development (days to pupation and eclosion) were approximately two and a half times greater when larvae were exposed to a combination of Bt anthers and pollen than when exposed to either tissue separately (Fig. 3). Evidence of additive effects also could be seen in larval weight (Fig. 3) and survival to pupation with tropical milkweed.

In both cage studies, significant effects were detected between larvae exposed to Bt pollen and those exposed to non-Bt pollen. Levels of pollen in our cage studies were below the no-observable-effects level reported by Hellmich et al. (2001) and were similar to levels tested in the petri dish studies, where no effects were detected between pollen-only treatments. Anthers and pollen in the petri dish studies had been stored for ≈6 mo at −20°C before they were used. A study by Jesse and Obrycki (2000) concluded that low concentrations of Bt protein in their pollen samples may have been caused by storing the samples at −20°C for 8–9 mo. Anthers and pollen used in the cage studies were collected and used the same day. Consequently, the concentration of Bt toxin was likely greater in the anthers and pollen in the cage studies than in the petri dish studies. Also, intact leaves used in the cage studies likely had higher levels of latex and cardiac glycosides than the leaf disks cut from severed leaves used in the petri dish studies (Dussourd 1993). Several studies have shown that cardiac glycosides and latex pose physiological costs to early instars (Zalucki and Brower 1992, Zalucki and Malcolm 1999, Zalucki et al. 2001).

The number and degree of adverse effects detected were greater with tropical than common milkweed. Differences were most likely caused by species characteristics such as leaf size and thickness or how the plants were presented to the larvae, either intact (tropical) or cut (common). Common milkweed leaves have greater mass and area than tropical milkweed. To consume an equal volume of leaf material, larvae must consume a larger area of a tropical leaf, which would result in larvae consuming more pollen and encountering more anthers. Based on leaf area data, when five anthers were placed on every leaf, larvae on tropical milkweed were being exposed to a density of 0.14 anther/cm^2, whereas larvae on common milkweed were only being exposed to 0.09 anther/cm^2. Also, as discussed in the petri dish studies, cutting the plant may have reduced the amount of latex and cardiac glycosides present in the plant, resulting in less adverse effects (Zalucki and Brower 1992, Dussourd 1993, Zalucki et al. 2001, Zalucki and Malcolm 1999).

Anther and pollen densities tested in the common milkweed cage study, five anthers per leaf and ≈200 pollen grains/cm^2, reflect the mean anther and pollen densities occurring on milkweed leaves in corn fields during anthesis (Pleasants et al. 2001, Anderson et al. 2004). The mean pollen densities tested in a recent risk assessment study by Dively et al. (2004) were similar; however, the anther densities were lower (1.1–1.8 anthers/leaf). Despite differences in anther density, the effects on pupal weight and developmental time seen in the common milkweed cage study were similar to those found by Dively et al. (2004).

Even though sublethal effects (decreased pupal weight and increased developmental time) were found with exposure to Bt anthers and pollen, the overall risk of Bt corn to monarch butterflies remains low. Risk is a function of the probability of toxicity and exposure. Toxic effects remained similar to those found previously even when anther densities were increased two- to three-fold. The prob-

ability of exposure also remains low. Components that factor into the calculation of the probability of exposure include the proportion of land in the monarch breeding range that is planted to corn, the proportion of that corn that contains lepidopteran-active Bt toxin, and the proportion of overlap between pollen and anther shed and susceptible stages of the monarch butterfly. These proportions remain low regardless of toxicity. As calculated by Dively et al. (2004), in a worse case scenario, assuming both direct effects on mortality and sublethal effects (e.g., reduced weights and increased developmental times), only 2.4% of the breeding population in the Corn Belt would be at risk from Bt corn. The population outside the Corn Belt (50% of the total population in North America) would be essentially unaffected by Bt corn (Wassenaar and Hobson 1998, Dively et al. 2004).

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